

# Determination of total polyphenolic compounds and flavonoids in *Juglans regia* leaves

Muhammad Nasimullah Qureshi<sup>1\*</sup>, Guenther Stecher<sup>2,3</sup> and Guenther Karl Bonn<sup>2</sup>

<sup>1</sup>Department of Chemistry, Abdul Wali Khan University, Mardan, Pakistan

<sup>2</sup>Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University Innsbruck, Innrain, 6020 Innsbruck, Austria

<sup>3</sup>Bionorica Research GmbH, Mitterweg 24, 6020 Innsbruck, Austria

**Abstract:** *Juglans regia* leaves have been widely used in traditional medicines because of its antimicrobial, antihelmintic, astringent, keratolytic, antidiarrhoeal, hypoglycaemic, depurative, tonic, carminative activity. Total polyphenolic compounds were determined using the Folin-Ciocalteu method and flavonoids were quantified using the HPLC-PDA after the hydrolysis of the plant material with HCl. Among the flavonoids myricetin, quercetin, apigenin and kaempferol were found in appreciable amount.

**Keywords:** *Juglans regia*, polyphenolic compounds, flavonoids, HPLC-PDA.

## INTRODUCTION

Plants have been proven to be the reservoir for various chemical compounds of biological and pharmacological importance (Qureshi *et al.*, 2011a; Qureshi *et al.*, 2013; Qureshi *et al.*, 2012; Qureshi *et al.*, 2011b; Sultana *et al.*, 2008). *Juglans regia* leaves have been widely used traditional medicines because of its antimicrobial, antihelmintic, astringent, keratolytic, antidiarrhoeal, hypoglycaemic, depurative, tonic, carminative activity (Taha and Al-wadaan, 2011). The leaves of English walnut have also been used for the treatment of sinusitis, cold and stomach ache (Girzu *et al.*, 1998; Mouhajir *et al.*, 2001) to reduce fever and rheumatic pain (Fujita *et al.*, 1995; Yesilada, 2002). It is a remedy for dermal inflammation, excessive perspiration of the hands, feet and for the treatment of chronic eczema and scrofula (Taha and Al-wadaan, 2011). The leaves are applied for the topical treatment of scalp itching and dandruff, sunburn and superficial burns as well as an adjunctive emollient in skin disorders (Ali-Shtayeh and Abu Ghdeib, 1999; Baytop, 1999; Blumenthal, 2000; Gruenwald *et al.*, 1998; Robbers and Tyler, 1999). It also has high anti-atherogenic potential and a remarkable osteoblastic activity (Papoutsis *et al.*, 2008). A number of phenolic and poly phenolic compounds have been isolated which could be the base for its enormous health benefits. Among the polyphenolic compounds flavonoids constitute a major class of bioactive compounds.

Flavonoids are present in nature mostly in the form of sugars conjugates mainly of glucose, rhamnose and rutinose (Herrmann, 1988) or as aglycones (Markham, 1982) but some time as aglycon. Flavonoids are mainly present in the leaves and outer parts of the plants. The flavonoid group is sub divided into many sub groups; the six main sub groups are flavonols, flavones, flavanols,

isoflavones, flavanones and anthocyanidins. There is a large number of flavonol glycosides present in plants. Flavones also occur as glycosides but the range of different glycosides is less than in the case of the flavonols. The presence of large number of glycosidic forms makes it difficult to determine their concentrations in plants. To overcome this problem the samples were subjected to acid hydrolysis which cleaved the glycon moieties releasing the aglycones which were analysed by reversed phase HPLC-PDA (Häkkinen *et al.*, 1998; Hertog *et al.*, 1992; Nuutila *et al.*, 2002).

The present study is aimed at the qualitative and quantitative analysis of flavonoids in the leaves of *Juglans regia*. The study specifically focuses on quantification of total polyphenolic compounds and quantification of flavonoids by HPLC using photodiode array detector (PDA).

## MATERIALS AND METHODS

### *Chemicals and reagents*

Acetonitrile, ethanol absolute, formic acid, methanol and THF were purchased from Merck KGaA (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent 2 N, HCl (37%), and phosphoric acid (85%) were obtained from Sigma Aldrich (Steinheim, Germany). Sodium carbonate and tetra-butylhydroxyanisole (t-BHA) were procured from Fluka Biochemika (Buchs, Switzerland). Water purified by a Nano Pure-unit (Branstead, Boston, MA, USA) was used. All these chemicals and reagents were of analytical grade and used without further purification. Standards gallic acid ( $\geq 97\%$ ), quercetin dihydrate (min. 98%) were purchased from Sigma-Aldrich. Apigenin ( $\geq 95\%$ ), kaempferol ( $\geq 96\%$ ), myricetin ( $\geq 95\%$ ) were obtained from Fluka Biochemika. All plants materials were provided by Bionorica AG – a phytoengineering company GmbH (Neumarkt, Germany).

\*Corresponding author: e-mail: mnasimuq@yahoo.com

### **Extraction and determination of total polyphenolic compounds**

Polyphenolic compounds were extracted by refluxing 1 g of powdered plant material in 20 mL of 50% methanol for 2 hours. Extracts were allowed to cool at room temperature and then centrifuged for 10 minutes at 14×1000 g using eppendorf centrifuge (Eppendorf 5415 D, Hamburg, Germany). They were preserved at -20°C temperature for further work.

### **Preparation of standards**

Stock solution of gallic acid was prepared with a concentration of 0.25 mg/mL in double distilled water. Further, working standard solutions were made in the concentration range (0.01 mg/mL – 0.2 mg/mL) diluting stock solution with double distilled water. Six calibration standards were made; each of which was measured thrice a time.

### **Folin-Ciocalteu method**

Polyphenolic compounds were determined by Folin-Ciocalteu method using gallic acid as the reference standard (Singleton and Rossi, 1965). 5 mL of diluted FC reagent (1:10 FC reagent to water) was added to 1 mL of each of standards, extract and blank (water) in test tubes. These were mixed thoroughly through vortex mixing. 4 mL of Na<sub>2</sub>CO<sub>3</sub> solution (7.5%) were added to each mixture after 8 minutes and mixed thoroughly through vortex mixing. These test tubes were covered and stored for 2 hours at room temperature and away from strong light. Absorbance of these test solutions were read against the prepared blank at 740 nm using UV-visible spectrophotometer (UV 2000, Hitachi).

### **Quantification of flavonoids**

#### **Preparation of calibration standards**

Stock solutions of the four standards apigenin, kaempferol, myricetin and quercetin were prepared in 50% methanol in the concentration range 0.255 mg/mL – 2.02 mg/mL and stored at -20°C until use. Working standard solutions for calibration curve were made by diluting stock solutions with 50% methanol to the desired concentrations (apigenin 0.053 mg/mL – 0.315 mg/mL, kaempferol 0.05 mg/mL – 0.302 mg/mL, myricetin 0.01 mg/mL – 0.061 mg/mL and quercetin 0.01 mg/mL – 0.505 mg/mL). Six calibration standards were made, each of which was measured thrice a time.

### **Extraction and hydrolysis**

Quantification of flavonoids aglycon in plant extracts was performed after subjected them to simultaneous extraction and hydrolysis. Simultaneous extraction and hydrolysis of the herbs was performed according to the hydrolysis procedure as published by Hertog *et al.* with some changes (Hertog *et al.*, 1992). 20 mL of 62.5% of aqueous methanol containing 2 g/L of tert-butyl hydroxyanisole (t-BHA) was added to about 1 g powdered plant material in

a round bottom flask. 5 ml of HCl (6 M) was added and mixed carefully. The mixture was refluxed at 90°C with continuous stirring using six place heating carousel reaction station. Extraction was performed for 2 hours. Extracts were let to cool at room temperature and centrifuged for 10 minutes at room temperature.

Extract with out hydrolysis was also prepared by refluxing 1 g of plant material in 20 ml of 50% methanol for 2 hours.

### **Instrumentation**

Shimadzu HPLC was used for the quantification of flavonoids. The HPLC system comprised of an online degasser unit (DGU-14A), two solvent delivery pumps (LC-10Advp), an autoinjector (SIL-10Advp), a column oven (CTO-10Avp) and a system controller (SCL-10Avp). Detection of the analytes was performed using a photo diode array detector PDA (SPD-M10 Avp). The system control and data analysis were performed using the manufacturer's software packages (LCMS-Solution, version 3 and LCMS-Post run, version 3-H2).

### **Chromatographic parameters**

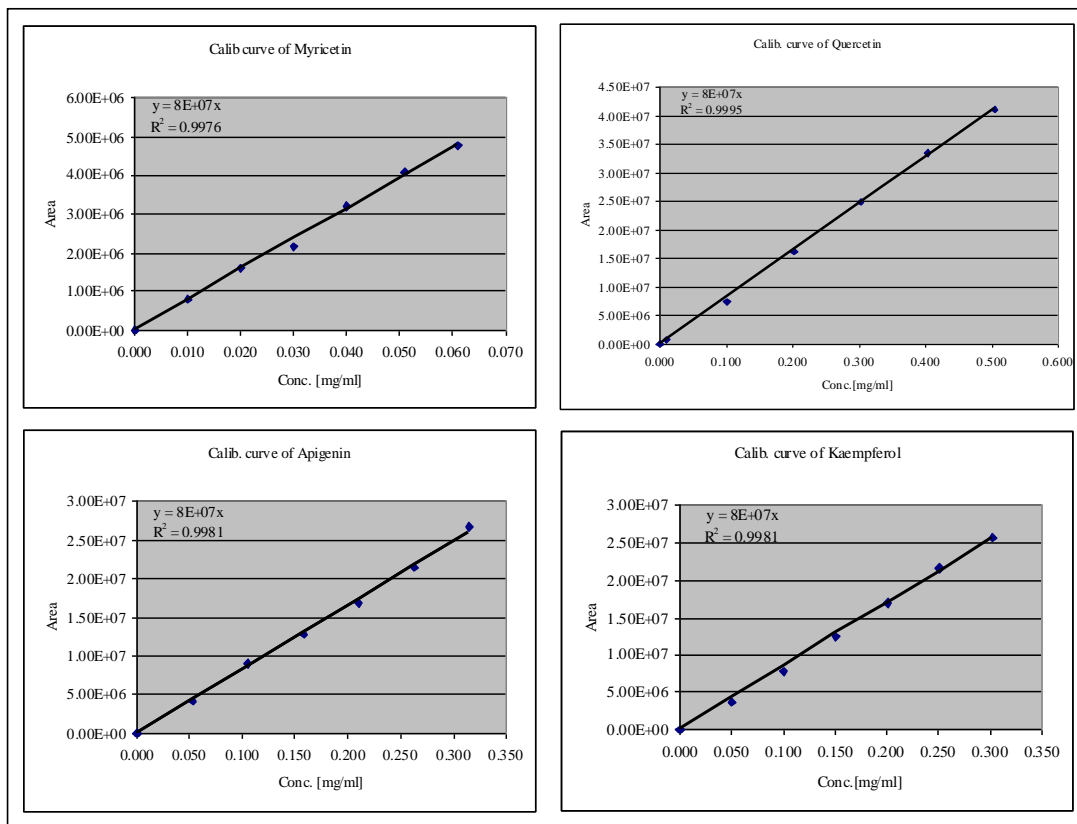
The chromatographic separation was performed on a reverse stationary phase column (Hypersil BDS 125×4 mm, 3 µm particle size and 130 Å pore size). Gradient elution was carried out using mobile phase A: 900 ml water+100 ml methanol+10 ml phosphoric acid (85%) and B: 600 ml water+300 ml tetrahydrofuran (THF)+100 ml methanol+10 ml phosphoric acid (85%). Column temperature was 50°C and a flow rate of 0.5 ml per minute was used. Zero time condition was 10% B and a linear gradient to 60% B was applied up to 10 minutes. Then elution was made isocratic at 60% B for 35 minutes and after that a linear gradient to 100% B up to 53 minutes was applied. The column was equilibrated at 100% B for 3 minutes and then changed to the zero time conditions in 4 minutes. 50% methanol was used as washing solution for the auto injector and the sample injection loop. The whole analysis took 70 minutes. PDA detector was operated in the wave length range of 200 to 600 nm.

## **RESULTS**

### **Quantification of total polyphenolic compounds and flavonoids**

Qualitative and quantitative assessment of flavonoids in the leaves of *Juglans regia* were carried out focusing on quantification of total polyphenolic compounds and quantification of flavonoids by HPLC-PDA.

Total polyphenolic compounds obtained in the plant extract were 189.28 mg/g. Fig. 2 shows the HPLC-PDA chromatogram of the non-hydrolysed plant extract while figs. 3 show the HPLC-PDA chromatograms of plant



**Fig. 1:** Calibration curves of standards myricetin, quercetin, apigenin and kaempferol along with straight line equation and  $R^2$  values.

hydrolysed with HCl obtained at 370 nm. From the results it appeared that myricetin, quercetin, apigenin and kaempferol were quantified in the plant extract. Quercetin was found in appreciable amount, and leads over the other flavonoids found in *Juglans regia*. The amount (mg/mL) of myricetin, quercetin, apigenin and kaempferol obtained are: 0.023, 0.247, 0.003 and 0.029 respectively. The %RSD values obtained were: 3.65, 0.89, 8.04 and 0.86 for myricetin, quercetin, apigenin and kaempferol respectively.

## DISCUSSION

### *Quantification of total polyphenolic compounds and flavonoids*

Folin-Ciocalteu method for the determination of polyphenolic acid was used (Singleton and Rossi, 1965).

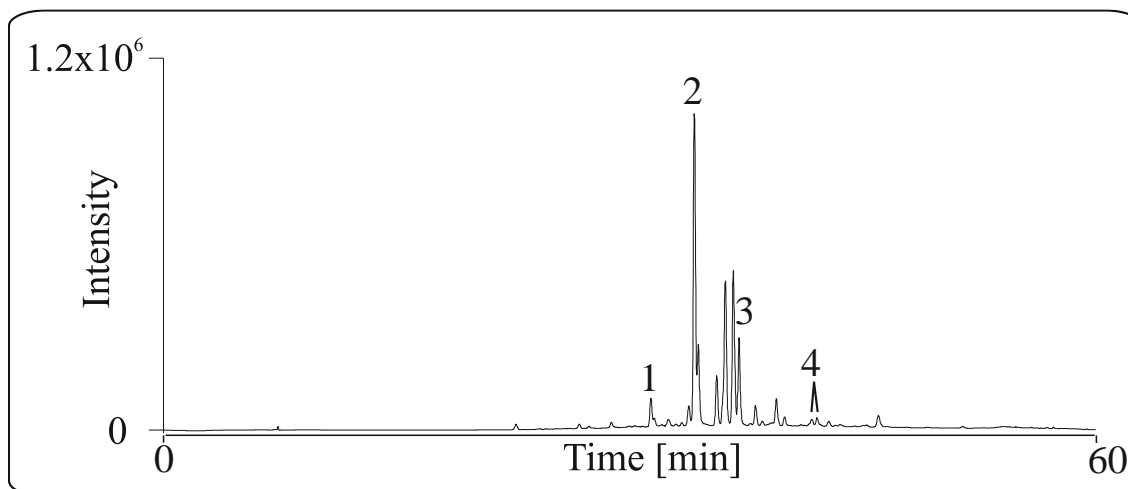
Because of the presence of large number of flavonoid glycosides, quantification of individual flavonoid glycosides are difficult. Additionally the availability and the cost of standards for these glycosidic forms of flavonoids also make it difficult to quantify these in complex plant extracts. These points make a strong base for the need of hydrolysis for these compounds.

Extraction and hydrolysis was performed simultaneously. As flavonoid glycosides are more soluble in water and flavonoid aglycon in methanol. (Hennig, 1980) Therefore, efficiency of the extraction depends on the water/methanol ratio. We used 50% methanol and the antioxidant tertiary butyl hydroxyanisole (t-BHA). Concentrations of the HCl in the final extracting media were 1.2 M.

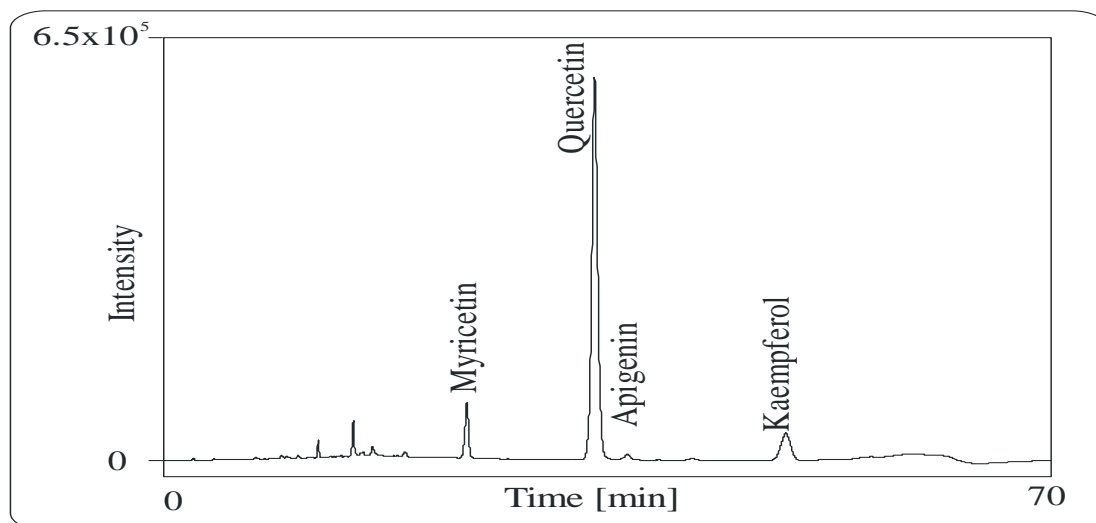
### *HPLC-PDA analysis for quantification of flavonoids*

RP-HPLC with photo diode detector (PDA) was used for the separation of the flavonoids aglycon. Preliminary separations of the flavonoids were performed using water and acetonitrile with 0.05% formic acid with different gradient timing. ProntoSil C-18 column with dimensions 250×4.6 mm and 5  $\mu$ m particle size was used. However, this did not enable base line separations between the flavonoids. Because of these disadvantages, a water – methanol – tetrahydrofuran with phosphoric acid as modifier was used as mobile phase as established by Stecher *et al.* (2001) Hypersil BDS column with dimensions 125 mm×4 mm with particle size 3  $\mu$ m (pore size 130 angstroms), column temperature of 50°C and a flow rate of 500  $\mu$ L / min was used. This system gave the best base line separation as well as peak purity, peak

shape and minimum peak tailing. The peak tailing shows calibration curves obtained for four flavonoid



**Fig. 2:** HPLC-PDA chromatogram of non hydrolyzed extract of *Juglans regia*. 1: Myricetin glucoside, 2: Quercetin glucoside, 3: Quercetin rhamnoside, 4: Kaempferol compound.



**Fig. 3:** HPLC-PDA chromatograms for quantification of flavonoids in hydrolysed plant extracts of *Juglans regia*. Chromatographic conditions: Mobile phase A: 900 mL H<sub>2</sub>O+100 mL MeOH+10 mL H<sub>3</sub>PO<sub>4</sub> 85% and B: 600 mL H<sub>2</sub>O+300 mL THF+100 mL MeOH+10 mL H<sub>3</sub>PO<sub>4</sub> 85%, Inj. Vol: 10 µL, Flow rate: 0.5 mL/min, Column Temp.: 50°C.

depends upon the use of different modifiers. When formic acid was used instead of phosphoric acid, the peak tailing increased. The order of elution was from most polar myricetin to least polar kaempferol that is myricetin, quercetin, apigenin and kaempferol respectively. Wavelength maxima for all the analytes investigated were determined as 376 nm, 373 nm, 340 nm and 368 nm of myricetin, quercetin, apigenin and kaempferol respectively.

Quantification of the four flavonoids myricetin, quercetin and kaempferol were made by measuring their absorbance in plant extracts at 370 nm, for apigenin at 340 nm. Fig. 1

standards. Six points calibration curve for each standard were made and the R<sup>2</sup> values obtained were 0.9976, 0.9995, 0.9981, and 0.9981 for myricetin, quercetin, apigenin and kaempferol respectively. The calibration curve was forced through zero in each case. Highest amount of quercetin was obtained among the flavonoids quantified in the plant extract of *Juglans regia*.

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